Methods for Predicting *In Vivo* Pharmacokinetics Using Data from *In Vitro* Assays

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Abstract: Strategies for optimising *in vivo* predictions from *in vitro* data on metabolic stability and CYP inhibition are discussed. Potential pitfalls and areas of inaccuracy are highlighted together with recommendations for best practice. The use of both hepatic microsomes and isolated hepatocytes for the assessment of metabolic stability is discussed in terms of scaling from the *in vitro* system up to whole liver. The importance of integrating metabolic stability data together with other drug pharmacokinetic characteristics (e.g., protein binding and red blood cell uptake) as well as blood flow are presented within the context of different liver models. The assessment of CYP inhibition potential requires *in vitro* data on the inhibitor potency either in the form of K_i (for reversible inhibition) or K_I and k_{inact} (for time-dependent inhibition). The integration of these *in vitro* parameters together with other pharmacokinetic information is essential for the *in vivo* prediction. While a qualitative assessment may be made from the I/K_i ratio, a number of additional victim drug and enzyme-related parameters are required for quantitative prediction. Of particular importance is the parameter fm_{CYP} (the fraction of the metabolic clearance of the victim drug that is catalyzed by the enzyme subject to the inhibition). Impact of other victim drug properties (e.g., fractional importance of the intestine) and enzyme properties (e.g., k_{deg} for time-dependent inhibition) on the drug-drug interaction prediction is discussed. In addition, mechanisms by which false negatives and false positives may result from *in vitro* strategies are summarized. Finally perspectives for future application and improvements in these predictions strategies are outlined.

Keywords: Clearance prediction, assessment of CYP inhibition potential, quantitative prediction of drug-drug interactions, *in vitro-in vivo* extrapolation.

INTRODUCTION

Previous articles in this special issue on *In vitro ADME/Tox profiling for Drug Discovery* have discussed the advances in *in vitro* techniques to obtain drug kinetic parameters. This article discusses how to make optimal use of this information, indicates potential pitfalls and areas of inaccuracy together with some recommendations for best practice. Both metabolic stability and assessment of CYP inhibition potential are discussed. Topics covered include - what *in vivo* information needs to be predicted, theoretical considerations for obtaining the necessary *in vitro* information i.e. its suitability for purpose, practical implications and databases of published literature illustrating application.

The principles of scaling *in vitro* parameters and modelling of CL_{int} and inhibition effects are well accepted due to their sound kinetic basis and their validation, to varying degrees, using rat tissue [1-3]. The use of this animal species, in contrast to human, allows consistency in both genetic components and environmental conditions and hence ensures maximum comparability between the experimental conditions applicable to the *in vivo* and *in vivo* phases of the studies. Several challenges remain to our ability to fully interpret human *in vitro* data and hence there remain uncertainties in our predictions despite our technical capabilities to readily generate *in vitro* parameters. This review summarises the current status of the methodology available for the use of *in vitro* systems for the prediction of human *in vivo* drug clearance and inhibition interaction potential.

ASSESSMENT OF METABOLIC STABILITY

There are two approaches available for the *in vitro* assessment of metabolic stability, the first involves measurement of the depletion of drug concentrations and the second involves measurement of the formation of specific metabolites. Both are monitored over time to allow a linear rate to be determined. The former is more widely used as in many cases the full metabolic profile is not available for the drug under investigation or, if indeed it is, authentic standards of those metabolites are not. However as discussed below the theoretical basis for extrapolation originates from metabolite formation kinetics and biochemical principles.

Both isolated hepatocytes and hepatic microsomes have been advocated as suitable sources of kinetic parameters, [4] from either metabolite formation (V_{max}, K_m and clearance) [5, 6] or drug substrate depletion (clearance or half-life) [7, 8]. These terms are interrelated in the following way to provide intrinsic clearance (CL_{int}):

$$CL_{int} = \sum_{i}^{n} \left(\frac{V_{max,i}}{K_{m,i}} \right) = \frac{0.693xV}{in\ vitro\ t_{1/2}} \quad (1)$$

where V_{max} and K_m represents the metabolite formation parameters for a specific metabolic pathway (*i*) and V is incubation volume. The pharmacokinetic parameters associated with drug elimination *in vivo* are clearance and half-life. However for many drugs neither of these parameters provides a true metric for the efficiency of the drug elimination process. Clearance from plasma concentration time profiles (via an AUC determination) must be factored to provide the metabolic component (i.e. remove the renal or any other non metabolic contributions). The term metabolic or hepatic (as it is assumed that the liver represents the dominant organ of metabolism) clearance, and its derivatives described below, are the best measures of drug elimination from an *in vivo* stance. Plasma half-life is a secondary parameter reflecting both the distribution characteristics as well as clearance of a drug. Therefore it is very limited value in providing a useful measure of drug elimination and as an *in vivo* correlate.

Theoretical Considerations Pertinent to *In Vitro* Studies

From a biochemical perspective, CL_{int} can be expressed in terms of the kinetic parameters of the one-site enzyme model defined by Michaelis-Menten equation. The free (unbound to macromolecules) concentration of drug within the liver is assumed to equate with the drug concentration at the enzyme site and under therapeutic conditions rarely approaches the K_m. Therefore linear conditions exist and the Michaelis-Menten equation reduces to Eq. 1.

The single-site Michaelis-Menten kinetic approach does not always accommodate the kinetic features observed for certain reactions [9]. A number of CYP3A4 and UGT substrates, for example, display kinetic behaviour that indicates the existence of and interaction between several binding sites on the enzyme [10-13]. Atypical

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Michaelis-Menten kinetics are characterised by two particular types of rate-substrate concentration curves - sigmoidal, believed to result from autoactivation, and convex, resulting from substrate inhibition [14]. Models describing these kinetic behaviours are available and have been discussed in previous reviews [1, 9].

It is important to address the practical problem of dealing with data that cannot be described by the Michaelis-Menten model with a view to making *in vivo* predictions, particularly as CYP3A4 is the major human cytochrome. Frequently these kinetic characteristics are regarded as solely a microsomal phenomenon. However, there are several reports in isolated hepatocytes supporting its *in vivo* importance [6, 15-17]. The initial steps of any *in vitro-in vivo* scaling strategy require full description of the *in vitro* data prior to abstracting useful parameter(s) for extrapolation. Thus the *in vitro* system kinetics needs to be characterized comprehensively, extending beyond the limits that may be observed *in vivo.* For substrate inhibition, a substantial underestimation of V_{max} will occur by merely ignoring the high concentration data points and forcing a standard Michaelis-Menten model through the remaining lower substrate concentration data. Also K_m would be poorly estimated. For a sigmoidal curve, there may be either an underestimation or overestimation of CL_{int} if a hyperbolic curve is forced through the data and the parameters V_{max} and K_m are used to calculate CL_{int}. How precisely the clearance estimate will be altered by the model misspecification will vary from case-to-case and will be dependent on the number and quality of the data points [9, 14].

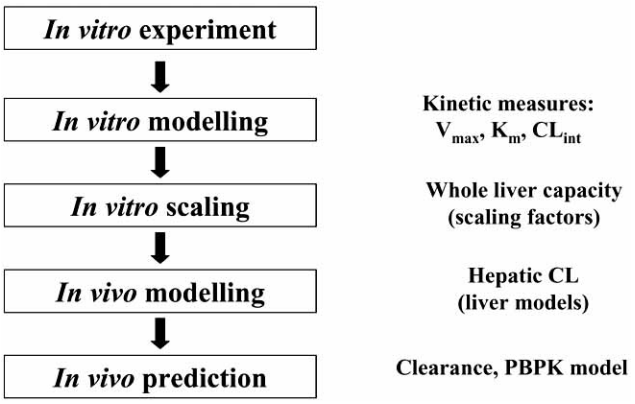
Atypical kinetics may impact on the use of substrate concentration depletion time profiles to obtain clearance values. This is usually carried out at one substrate concentration, often in the 1μM region, based on the rationale that this concentration should be well below the (unknown) K_m value. If consideration is not given to the phenomenon of activation then underestimates of clearance will occur. For example, diazepam metabolite kinetics shows marked sigmoidicity and its microsomal half-life at 1μM is five times longer than at 100μM reflecting the nonactivated and fully activated clearance [14]. In contrast the phenomenon of substrate inhibition *per se* is unlikely to be of consequence *in vivo* due to the high concentrations required. The introduction of CL_{max} as an alternative for CL_{int} represents one attempt to introduce autoactivation into the *in vitro-in vivo* scaling strategy. Autoactivation is characterized by the absence of any clearance independent of substrate concentration; CL_{max} reflects clearance when the enzyme is fully activated [9].

The need to incorporate the fraction unbound in microsome (fu_{inc}) to obtain meaningful drug concentrations for the prediction of intrinsic clearance is widely accepted [18-20]. Recently, two equations based on drug lipophilicity have been developed for prediction of fu_{mic} [21, 22] which avoid experimental determinations. The limitations of these empirical predictive tools and their applicability for fu_{mic} predictions over a range of lipophilicity and microsomal protein concentrations have been addressed [23]. Analogous to applying a correction for microsomal drug binding to *in vitro* clearance and inhibition parameters, the fraction unbound in hepatocyte incubations (fu_{hep}) is also required for *in vitro-in vivo* extrapolation [5, 24]. However, this need has yet to be broadly applied. The relationship between the extent of binding in microsomal and hepatocyte incubations has been used as an empirical tool for the prediction of fu_{hep} directly from the drug lipophilicity metric or from a previously derived fu_{mic} value [25]. The use of these calculated unbound fractions, while far from precise in many cases, are preferable to ignoring this important phenomenon.

QUANTITATIVE PREDICTION OF METABOLIC CLEARANCE

The general strategy adopted (Scheme **1**) conceptually consists of four stages, yet in practical terms this amounts to two essential steps. The first step expresses the *in vitro* clearance parameter (CL_{int} initially obtained by appropriate modelling of the *in vitro* data) in terms of total liver weight rather than the *in vitro* units of *per* million cells or *per* mg of microsomal protein. CL_{int} is a pure measure of enzyme activity towards a drug. The second step incorporates physiological processes (such as hepatic blood flow or drug binding within the blood matrix) with the intrinsic metabolic stability of the drug to provide a whole liver clearance parameter (CL_h). Other clearance terms may be required in order to obtain total body clearance, such as renal or biliary excretion of unchanged drug [26].

General strategies for prediction from *in vitro* kinetic data: Clearance



Scheme 1.

Step One, Scaling *In Vitro* Parameters

The output from an *in vitro* experiment should be the intrinsic clearance expressed as μL per minute per milligram of microsomal protein (or per million cells). Hence the parameter will be linear not only with respect to time but with concentration of enzyme (as measured indirectly by either milligram of protein or hepatocellularity). These units allow the intrinsic clearance term to be scaled to the whole liver in a convenient way as exemplified in the case of the hepatocytes using a hepatocellularity value per gram liver or per entire tissue weight. Commonly employed values for hepatocellularity are very similar in human and rat (see Table **1**). For microsomal scaling a similar procedure is carried out; however, it is important to appreciate that the isolation of microsomes is not an efficient process. Therefore, the microsomal scaling factor (milligrams of microsomal protein per gram liver) is not equivalent to the microsomal protein content; rather it reflects the efficiency of the microsomal preparation. Values commonly used for rats and human tissue are shown in Table **1**. Recently a consensus paper on the value necessary for human microsomal scaling has been published [27].

The use of other sub-cellular fractions, for example cytosol, will require distinct scaling factors. Table **1** lists values useful for cytosolic enzymes, such as sulphotransferase. To date the use of S9 fraction has not been extensively explored. Also, for the use of *in vitro* data from extra hepatic tissues, tissue specific scaling factors will be necessary to achieve measures for the entire organ. Limited literature indicate that protein yield from kidneys is similar to that of liver whereas from intestinal mucosa microsomal scaling factors are substantially lower.

Whereas establishing a linear relationship between both time and enzyme concentration is a standard procedure for metabolite formation studies, it is less commonly explored for drug depletion. Particularly as in most laboratories generic protocols are employed in order to take advantage of high-throughput screening procedures. It has recently been demonstrated that linearity in terms of enzyme concentration (as reflected by microsomal protein concentration or hepatocellularity) is not always predictable and recommendations

Table 1. Key Parameters for Scaling *In Vitro* Data to Predict *In Vivo* Clearance. Values are shown for microsomes and hepatocytes from both rat and human. In addition, bias in predictions using physiologically based scaling factors are shown.

	Rat	Human[a]	Reference
Microsomal recovery (mg protein /g liver)	45	40 (13-54)	[4, 18, 27, 87]
Hepatocellularity (10^6 cells/g liver)	120	99 (74-131)	[4, 27]
Cytosolic recovery (mg protein /g liver)	91 (78-105)	80.7 (45-134)	[88-96]
Q_H mL/min/SRW or mL/min/kg[a]	25	20.7 (17.1-25)	[18, 28, 97]
Bias in CL prediction[b]			[5, 18, 28]
Using hepatocytes	1.4	4.5	
Using microsomes	2.2	9.2 (well stirred model) 6.3 (parallel tube model)	

[a]Values represent the weighted mean and the range is quoted in brackets. [b] Data shown represent fold under-prediction (using the well stirred liver model unless specified).

have been made for appropriate enzyme concentrations for each system [7].

Step Two, Use of Liver Models

This section on the use of physiological based modelling for clearance prediction is limited to the liver as this organ is almost exclusively investigated. Scaled CL_{int} terms need to be refined further in order to allow comparison between *in vitro* and *in vivo*. Two considerations are important; first, plasma protein binding and red blood cell uptake (requiring measures of unbound plasma fraction and the blood plasma concentration ratio) and secondly, any possible blood flow limitations (becoming increasingly important as clearance increases). In order to achieve this it is necessary to use a physiologically-based pharmacokinetic model (so called liver models). By applying a liver model we can both isolate the *in vivo* intrinsic clearance term (a pure measure of drug metabolic instability) and model the *in vivo* hepatic clearance using *in vitro* input.

Comparison between *in vivo* and *in vitro* metabolism rates can be made both at the level of hepatic clearance and intrinsic clearance. The latter has the advantage of having no numerical limits whereas the former will have an upper limit of hepatic blood flow (see Table **1** for values). In practise two approaches are adopted for *in vitro* and *in vivo* comparisons. For the *Intrinsic Clearance* approach, the *in vivo* AUC provides the value for total systemic clearance and subsequently hepatic clearance. This is deconstructed in order to give an *in vivo* intrinsic clearance, which in turn can be compared with in the *in vitro* prediction. For the *Hepatic Clearance* approach, the *in vitro* intrinsic clearance is modelled together with other parameters (plasma protein binding, blood/plasma concentration ratio and blood flow) in order to provide a prediction of hepatic clearance and then, if required, total systemic clearance. These predicted clearances can then be compared with the corresponding *in vivo* parameter. The *Hepatic Clearance* approach is more widely carried out within the pharmaceutical industry however the *Intrinsic Clearance* approach has more appeal from an academic view. It is recommended that comparisons of drug metabolic instability are made by both approaches.

There are 3 established liver models: the 'well-stirred', parallel tube and dispersion models. The former model is most often used due to its mathematical simplicity rather than any superiority over the others, indeed it is the least 'physiological' in nature. To mimic the *in vivo* situation these models all assume that (1) the distribution into the liver is perfusion rate limited with no diffusion barriers, (2) only unbound drug crosses the cell membrane and occupies the enzyme site (the free drug hypothesis), and (3) there is a homogenous distribution of metabolic enzymes in the liver [4]. However, different assumptions are made regarding the concentration gradient of drug within the liver: the 'well-stirred' model assumes that the hepatic drug concentration is equal to the outflow concentration, the parallel tube model assumes it is equal to the logarithmic mean of inflow and outflow concentration and the dispersion model assumes it shows axial dispersion analogous to packed-bed chemical reactor.

The relationships between intrinsic clearance, blood flow (Qh) and blood binding (fu) for the 3 models are shown in Eq. 2-4:

Well-stirred model:

$$CL_h = \frac{Q_h \cdot f_u \cdot CL_{int}}{Q_h + f_u \cdot CL_{int}} \quad (2)$$

Parallel tube model:

$$CL_h = Q_h\left[1 - \exp\left(-\frac{f_u \cdot CL_{int}}{Q_h}\right)\right] \quad (3)$$

Dispersion model:

$$CL_h = Q_h\left[1 - \frac{4a}{(1+a)^2\exp\left[(a-1)/2Dn\right] - (1-a)^2\exp\left[-(a+1)/2Dn\right]}\right] \quad (4)$$

where $Dn = 0.17$, $a = \sqrt{1+4RnDn}$ and $Rn = \frac{f_u \cdot CL_{int}}{Q_h}$

A recent comprehensive comparison of the use of liver models to predict *in vivo* CL_{int}, CL_h and hepatic availability was carried out using rat *in vitro* data in both hepatocyte and microsomal preparations [28]. When CL_{int} values from *in vitro* and *in vivo* are compared it is clear that the simpler 'well-stirred' model gives the poorest predictions and there is nothing to distinguish the dispersion and parallel tube models. Considering the mathematical complexity of the dispersion model, it is recommended that the parallel tube model be used in preference to the 'well-stirred' model for the prediction of *in vivo* CL_{int}. To validate a principle, the emphasis on CL_{int} is appropriate as this parameter provides the widest range of values (4 orders of magnitude) to allow *in vitro-in vivo* comparisons. However, often the aim of an *in vitro* study is to predict CL_h and/or hepatic availability. For predictions of these parameters (using either hepatocyte or microsomal data) there is less difference between the particular models based on statistical estimates of bias and precision and the common practice of using the 'well-stirred' model would appear to be quite satisfactory. Certainly for screening procedures to identify new chemical entities with low CL_h (or high availability) *in vivo*, the 'well-stirred' model can be incorporated into prediction algorithms without concern.

Databases of Literature Reports of Prediction

The prototype system illustrating the utility of *in vitro* data for prediction of clearance *in vivo* is freshly isolated rat hepatocytes [4,

29, 30]. Using data from several laboratories, hepatocellularity was shown to provide excellent predictions of *in vivo* intrinsic clearance covering four orders of magnitude. The predictions show no bias and the majority are within a two-fold error margin (see Table **1**). The success of this particular *in vitro* system lies in its intact cellular structure and full complement of drug metabolizing enzymes. Neither of these conditions exist in hepatic microsomes, the *in vitro* system most commonly used *in vitro* system for human tissue. However despite these inherent disadvantages rat studies have allowed a similar validation of the use of microsomes albeit with a bias more evident than with hepatocytes (Table **1**)**.**

Recently it has become clear that although the simple scaling approach described above provides good clearance predictions in rat [28] it results in a high incidence of under-prediction for human pharmacokinetic parameters [5, 18]. Figs. (**1** and **2**) illustrate microsomal and hepatocyte data collated from several laboratories and the utility of adopting the standard, physiologically-based scaling strategy. *In vitro* intrinsic clearances have been corrected for other microsomal or cellular binding using experimentally determined values or predicted values based on logP. A strong correlation is evident ($r^2 > 0.8$) over a range of CL_{int} covering four orders of magnitude however there is a bias in the predictions resulting in a systematic under-prediction (Table **1**). Many factors may contribute to this situation including inter-individual variability and the potential mismatch between liver donors providing tissue for the *in vitro* studies and subjects involved in the *in vivo* studies. How the choice/availability of donor tissue impacts on the apparent improvement in hepatocyte over microsomal predictions has yet to be clearly demonstrated.

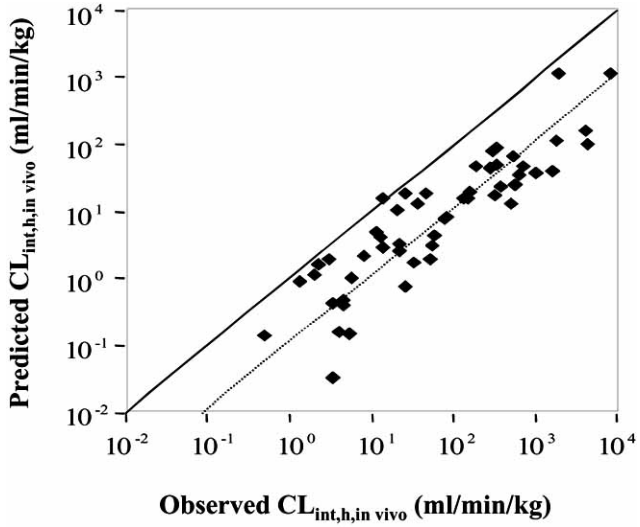


Fig. (1). Comparison between the observed *in vivo* human CL_{int} and CL_{int} predicted for a dataset of 52 drugs [18]. Predicted clearances were calculated from human hepatic microsomal data using a physiologically-based scaling factor of 40mg protein/g liver. The solid line represents the line of unity, whereas the dashed line represents the average fold under-prediction (9.2, using the well stirred liver model – Table **1**).

The general observation of under-prediction from human tissue raises the question of whether we can do better? Over the last few years several approaches have been advocated or re-assessed as alternatives to the use of *in vitro* human microsomal data to predict human drug clearance. Several make use of preclinical animal data and include the use of allometry [31], the incorporation drug specific factors (derived from the ratio of *in vivo*/*in vitro* CL_{int} in rats [32] and combinations of these together with *in vitro* human microsomal data [33]. In addition some investigators have suggested the possibility of empirical scaling (based on regression analysis from a historical data base) while others propose ignoring drug binding to plasma when applying a liver model. The basis for abandoning the free drug hypothesis is nonspecific binding within the microsomal matrix and hence the parameters describing the two binding processes may cancel out when a liver model is applied [34].

From a comprehensive comparison of all the above approaches it was concluded [18] that from the various alternatives to physiologically-based scaling of *in vitro* data available, only the empirical scaling approach removes bias and maintains precision. Thus an empirical scaling factor of 360 mg protein/g liver (9 times the physiologically-based scaling factor derived from microsomal recovery) has been proposed as a pragmatic solution to under-prediction. A scientific rationale is now required to support this proposal and to assure routine, reliable prediction of human clearance. There is a need to incorporate inter-liver variability into prediction strategies in order to account for the lack of comparability between tissue donors and healthy volunteers. Practically this can be achieved through the inclusion of internal standard of reference drugs in all incubation sets. If reference drugs are selected on the basis of well characterised *in vivo* parameters then novel *in vitro* data can be both ranked to these internal standards and normalised to *in vivo* activity empirically.

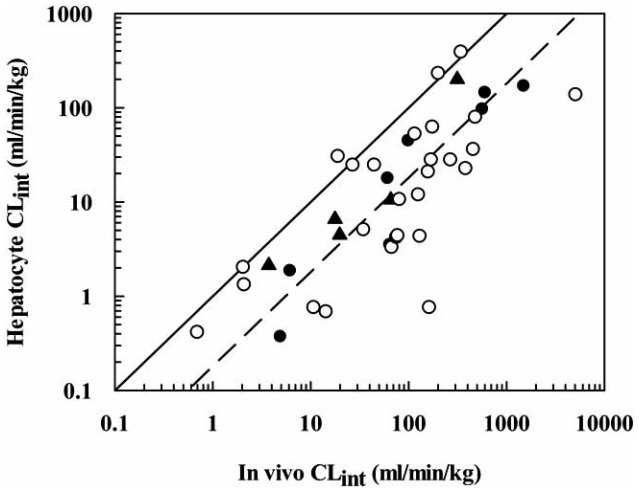


Fig. (2). Comparison between observed *in vivo* human CL_{int} and CL_{int} predicted using human cryopreserved hepatocytes for a dataset of 37 substrates [5]. The solid line represents the line of unity, whereas the dashed line represents the average fold under-prediction (4.5, using the well stirred liver model – Table **1**). Symbols denote sources of data [5].

ASSESSMENT OF CYP INHIBITION POTENTIAL

The use of *in vitro* data to delineate inhibition of CYP-mediated drug metabolism and hence identify potential serious drug-drug interactions (DDIs) in humans is widespread [35-42]. Over recent years, substantial technological advances have been made in the conduct of *in vitro* studies in terms of automation and generic protocols. However, there is still a lack of confidence in outcome, in particular in regards to false negative and positive predictions. The area is complicated by the existence of two major mechanisms responsible for inhibition of drug metabolism – reversible competitive inhibition and time-dependent (mechanism-based) inhibition.

The most common *in vivo* metric used to assess DDIs is the change in area under the plasma concentration time curve (AUC) of the victim drug following multiple dosing of an inhibitor relative to the control state [37, 38, 43, 44]. The AUC is the most reliable parameter as it inversely reflects clearance. It is assumed that the second drug (putative inhibitor or perpetrator) has reached steady state by the second phase of the study. Often the change in the clearance

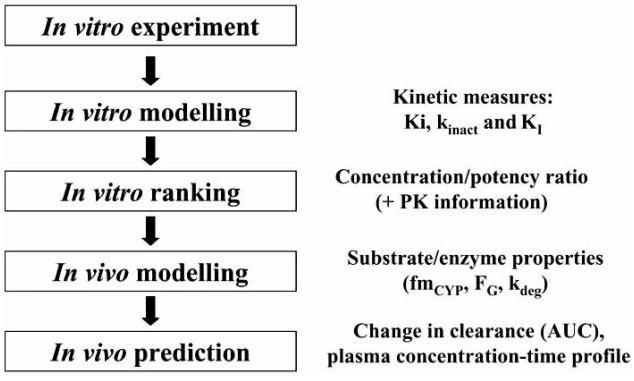
is related to the average steady state concentration of inhibitor during the dosing interval [36, 45]. In contrast to a time-averaged value, certain simulation programmes (for example Simcyp®) can incorporate the time course of the inhibitor concentration and hence generate a temporal profile of the inhibition process [20]. This is a valuable option when dealing with reversible inhibition mechanisms but it is less critical for time-dependent inhibition where the reduced state of activity is relatively stable, at least during the dosing interval period of study.

The prediction of DDIs occurring via inhibition of cytochrome P450 can be envisaged as a number of stages, similar to that discussed for clearance (Scheme **2**). Data from the *in vitro* experiment is first modelled using traditional biochemical models. The CL_{int} of substrate is reduced by a factor related to the inhibitor concentration available to the enzyme [I] and the inhibition constant, K_i (Eq. 5, where subscript I represents the clearance in the presence of inhibitor). The distinction between competitive and non-competitive inhibition mechanisms is not relevant when the substrate concentration is much lower than the K_m value, the commonly encountered *in vivo* situation that results in linear kinetics.

$$CL_{int,I} = \frac{CL_{int}}{1 + [I]/K_i} \quad (5)$$

This allows an *in vitro* ranking to be made provided that information from pharmacokinetic studies on circulation concentration of inhibitor is available, in addition to the *in vitro* inhibition data. This I/K_i rank order across different P450 enzymes helps in prioritising of the *in vivo* assessment, starting with the P450 with largest I/K_i and therefore strongest inhibition potential [40, 46]. This basic relationship between the AUC ratio and $[I]/K_i$, allows predictions to be categorized into four zones: true positives (AUC ratio>2, $[I]/K_i$ >1), true negatives (AUC ratio<2, $[I]/K_i$ <1), false positives (AUC ratio<2, $[I]/K_i$ >1), or false negatives (AUC ratio>2, $[I]/K_i$ <1) [38, 44]. In the subsequent steps the data are modelled to predict an interaction for a particular victim drug. At this stage, specific input of the victim drug-related parameters (e.g., fm_{CYP}, F_G) and the inhibited enzyme (e.g., k_{deg}), is required to allow the final prediction. Following the full strategy allows quantitative prediction; alternatively the strategy may be stopped after the second step and *in vitro* ranking used to qualitatively zone the inhibition information (see above).

General strategies for prediction from *in vitro* kinetic data: drug-drug interactions



Scheme 2

Qualitative Zoning of Reversible Drug-Drug Interactions

In recent years there has been much interest in the use of the ratio of estimated inhibitor concentration *in vivo* [I] and *in vitro* potency (defined by K_i) to describe the degree of *in vivo* interaction between two drugs [35, 37-39, 41, 42, 47]. The accurate determination of [I] is problematic as direct measurement is not possible and there is no generally accepted approach for the extrapolation of inhibitor concentration in the plasma to that at the enzyme site. A number of predictions of the drug-drug interactions have been attempted with varying degrees of success using a range of [I] values as a surrogate, including the average plasma total or unbound concentration or hepatic input concentration of the inhibitor [38, 48]. The hepatic input concentration combines the circulating systemic plasma concentration and the concentration of an inhibitor occurring during the absorption phase.

Comprehensive analysis of 193 drug-drug interactions involving inhibition of CYP2C9, CYP2D6 and CYP3A4 [38] has shown that hepatic input concentration was the most successful as a surrogate for [I] for categorising CYP inhibitors and for identifying true negative DDIs. False negative predictions were eliminated using this approach; however, significant number of false positives was evident and most true positives were markedly over-predicted (Fig. **3**). Although the $[I]/K_i$ ratio provides a useful tool in qualitative zoning and ranking of putative inhibitors [38, 40], this approach should be considered as an initial discriminating screen as it is empirical and requires subsequent mechanistic studies for comprehensive evaluation of a positive result [1]. This simple generic approach ignores the substrate- or inhibitor-specific properties that contribute to a number of over-predictions of true positive interactions. For example, in order to avoid false negative prediction and obtain the largest hepatic input concentration, maximum value of k_a (0.1 min^{-1}) was suggested as useful, assuming the gastric emptying is the rate limiting step for absorption [38, 48]. Recent studies by Brown *et al.* [47] have shown the impact of appropriate k_a values for 10 inhibitors for CYP2C9, CYP2D6 and CYP3A4, including azoles, quinidine, fluoxetine, fluvoxamine etc. Refinement of this parameter resulted in the k_a values 2- to 14-fold lower than the initial estimates and reduction of the relative contribution of the absorption term in comparison to the systemic term to the hepatic input concentration up to 13-fold in case of itraconazole. In addition, the k_a value may vary with the dose of inhibitor and the food intake (e.g., ketoconazole) [49], affecting the estimate of the inhibitor concentration and consequently the predicted AUC ratio.

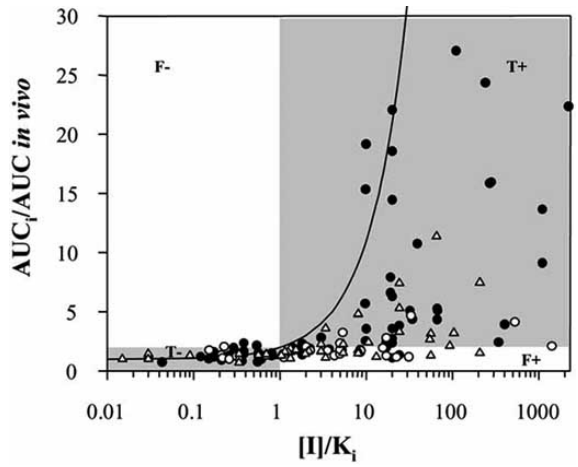


Fig. (3). Qualitative zoning for the prediction of drug-drug interactions involving CYP inhibition. The curve represents the theoretical curve based on the relationship AUC ratio = 1+ $[I]/K_i$, where [I] represents hepatic input concentration. F- represents false negative, T-, true negative, F+, false positive and T+, true positive DDI prediction. ● represents CYP3A4, △, CYP2D6 and ○ CYP2C9 DDIs [38].

Time-Dependent Drug-Drug Interactions

Irreversible effects, often referred to as mechanism-based or time-dependent inhibition interactions, involve the metabolism of an inhibitor (i.e., require NADPH) to a reactive metabolite, which inactivates the catalysing enzyme in a concentration- and time-dependent manner. Traditionally two major kinetic parameters are

used to characterize time-dependent inhibition interactions (TDI): k_{inact} and K_i, the maximal inactivation rate constant and the inhibitor concentration leading to 50% of k_{inact}, respectively [50]. The general approach to TDI *in vitro* risk assessment is based on the use of the k_{inact}/K_I ratio as an indicator of *in vitro* potency. Experimentally the two-step dilution method, although most commonly used [51-53], varies significantly in the design across the studies, from the CYP3A4 probes used (midazolam or testosterone), probe concentration (from below the K_m to the concentrations equivalent to V_{max}), pre-incubation to incubation time ratio to the data analysis method employed; all of which may affect the estimates of inhibitor potency [36, 54]. Ideally, pre-incubation to incubation time ratio should be at least >1, dilution factor from the pre-incubation to the incubation stage 1:10 should be applied to reduce the occurrence of competitive inhibition; in addition the use of high substrate concentrations (equivalent to V_{max}) and nonlinear regression analysis are recommended for obtaining the k_{inact} and K_I parameters. In addition, this approach assumes that the inhibitor concentration in the final incubation is negligible and therefore the rate of metabolism of a probe substrate represents the remaining enzyme activity; this may not be correct depending on the conditions mentioned above [55, 56].

Recently, the use of IC_{50} reduction over various pre-incubation times has received attention [39], as a potential alternative approach to the two-step dilution method. Although rapid, the application of this method is limited, in particular for inhibitors showing high potency for reversible inhibition that may obscure the time-dependent effect. In addition, significant depletion of an inhibitor over the pre-incubation time will result in a lack of significant decrease in the IC_{50} and a false negative result for a potent TDI. A third possible approach involves the use of the progress curve to monitor time-dependent inhibition [57]. This is the least explored method yet potentially it may offer solution to the limitations outlined above. It allows measurement of a probe substrate metabolism independently of the metabolism of an inhibitor. Wimalasena and Haines [58] have reported high reproducibility of the *in vitro* parameters obtained by this method in comparison to the two-step dilution assay. In particular, this method may be useful for investigation of potent inactivating inhibitors (e.g., mibefradil).

QUANTITATIVE PREDICTION OF DRUG-DRUG INTERACTIONS

Consideration of a number of factors, including existence of more than one metabolic/elimination pathway [36, 40, 47, 59], impact of multisite kinetics for CYP3A enzyme [1, 37], contribution of the intestinal inhibition [35, 39, 60, 61], impact of enzyme properties [36] and the role of multiple inhibitors and mechanisms [62, 63] is required in order to progress towards a quantitative basis. The impact of these factors on the magnitude of the predicted DDI is discussed in the following sections.

The full algorithm for quantitative prediction of AUC in the presence and absence of the inhibitor after multiple oral dosing is shown in equations 6 and 7 for reversible and irreversible inhibition interactions, respectively [38, 41, 60]:

$$\frac{AUC'}{AUC} = \frac{F_G'}{F_G} \cdot \frac{1}{\sum_{i}^{n} \frac{fm_{CYPi}}{1+\sum_{j}^{m}[I]_j/K_{i,j}} + \left(1-\sum_{i}^{n} fm_{CYPi}\right)} \quad (6)$$

where I_j is the estimated unbound inhibitor concentration (either the average systemic plasma concentration after repeated oral administration ($[I]_{av}$), or the maximum hepatic input concentration ($[I]_{in}$) [38, 48], $K_{i,j}$, is the particular inhibition constant, fm_{CYPi} represents the fraction of a substrate drug metabolised by the inhibited pathway via a particular P450 enzyme, ($1-\Sigma fm_{CYPi}$) represents clearance via other P450 enzymes and/or renal clearance; the terms *i* and *j* indicate the potential to incorporate existence of multiple enzymes and inhibitors, respectively. F_G' and F_G represent the intestinal wall availability in the presence and absence of the inhibitor, respectively.

An analogous relationship exists for irreversible inactivation, where the model also incorporates parameters to describe *in vitro* inactivation and *in vivo* enzyme degradation rate constant:

$$\frac{AUC'}{AUC} = \frac{F_G'}{F_G} \cdot \frac{1}{\sum_{i}^{n} \frac{fm_{CYPi}}{\left(1+\sum_{j}^{m} \frac{k_{inact,j} \times [I]_j}{k_{deg} \times (K_{I,j}+[I]_j)}\right)} + \left(1-\sum_{i}^{n} fm_{CYPi}\right)} \quad (7)$$

where $k_{inact,j}$ represents the maximal inactivation rate constant, $K_{I,j}$ the inhibitor concentration at 50% of $k_{inact,j}$ and k_{deg} the endogenous degradation rate constant of the enzyme [36, 61]. In addition to inhibition by multiple inhibitors, the model can easily be extended to accommodate the inhibition via different inhibition mechanisms (reversible and time-dependent) [41, 62].

Impact of Parallel Elimination Pathways

The most sensitive substrate-related parameter in the DDI prediction model is fm_{CYPi}, representing the fraction of the metabolic clearance of the victim that is catalysed by the enzyme subject to inhibition [37, 47, 59, 64]; in the qualitative analysis fm_{CYP} is assumed to be one.

Fig. (**4**) shows the importance of the introduction of the fm_{CYP} term in the DDI prediction model for reversible inhibition. Even minor changes in the fm_{CYP} value (e.g., from 1 to 0.98) alter prediction accuracy significantly, as was illustrated in recent studies [47, 59]. The inhibitory effect, i.e. the change in the AUC, decreases progressively with the decreasing contribution of the enzyme of interest to the overall elimination of a substrate. For victim drugs where enzyme contributes less than 50% to the overall elimination the AUC ratio will not exceed 2. It is clear that fm_{CYP} is of equal importance to the I/K_i ratio, as in some circumstances substantial increases in the latter parameter will reduce no further AUC change because of the limitation of the fm_{CYP} [36, 47, 59]. The systematic analysis of this parameters showed that incorporation of fm_{CYP} reduced significantly the extent of over-predictions of true positives and corrected several false positive predictions. The importance of fm_{CYP} in the DDI prediction model was confirmed further in the extensive analysis of 115 *in vivo* DDI studies for CYP2C9, CYP2D6 and CYP3A4 [45], where the incorporation of parallel pathways of substrate elimination with the *in vitro* inhibition data obtained under optimal standardized conditions substantially improved the prediction accuracy (Fig. **5**).

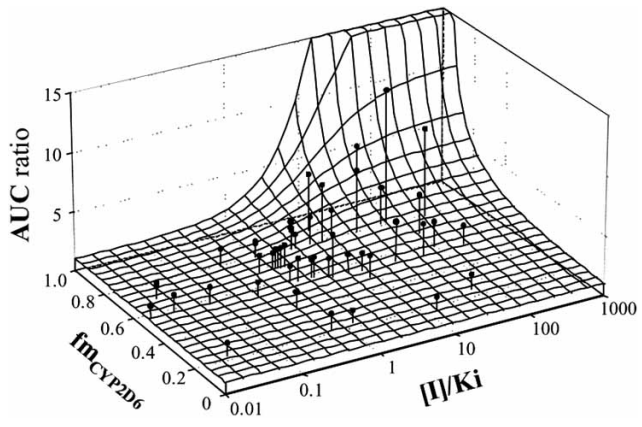


Fig. (4). Three-dimensional surface for the relationship between the AUC ratio, the $[I]/K_i$ and fm_{CYP2D6} for 44 drug-drug interaction studies involving CYP2D6 [59].

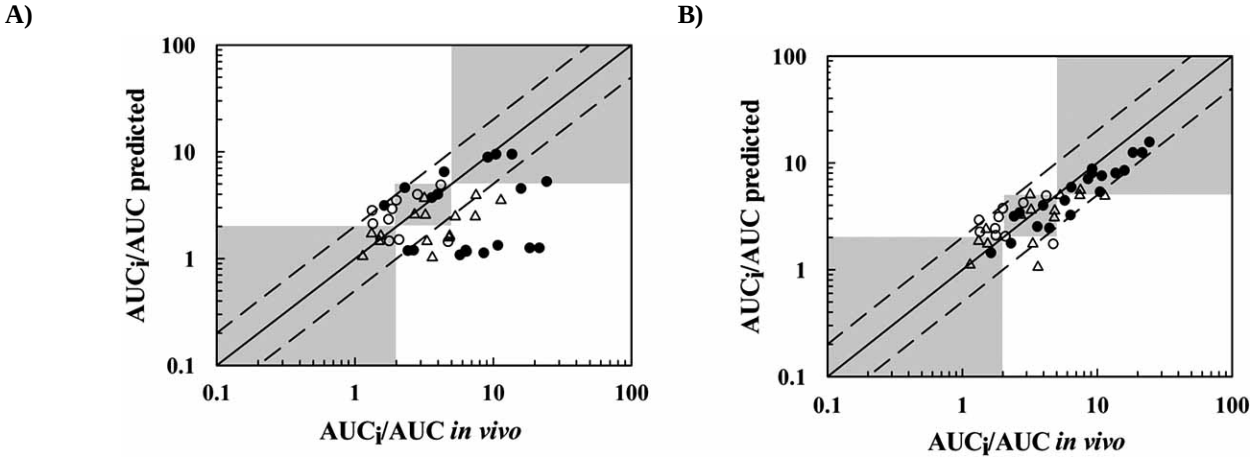


Fig. (5). Relationship between predicted and observed AUC ratios for reversible drug-drug interactions. Predicted AUC ratios obtained using the average systemic total drug plasma concentration, standardised *in vitro* data and incorporating fm_{CYP} for CYP2C9 (○), CYP2D6 (△) and CYP3A4 (●) in the drug-drug interaction database from [59] (A), and the corresponding studies in the database from Brown *et al.* [45] (B). The grey boxes shown relate to potent, moderate and weak DDI using the AUC ratio criteria [43].

Table 2. Estimated fm_{CYP3A4} values for 28 CYP3A4 Substrates. Contribution of renal clearance or metabolism via other CYP enzymes is indicated.

Victim drug	Estimated fm_{CYP3A4}	Reference
Alprazolam[a]	0.8	[46]
Atorvastatin	0.77	unpublished data
Buspirone	0.99	[35]
Carbamazepine	0.6	[35]
Cerivastatin[b]	0.39	[36]
Cisapride	0.95	[35]
Cyclosporine	0.71	[35]
Felodipine	0.81	[46]
Loratadine	0.6	[35]
Loperamide[b]	0.53	[61]
Lovastatin	0.90	[85]
Midazolam[e]	0.94	[46]
Nifedipine	0.71	[46]
Nitrazepam	0.6	[35]
Pimozide[c]	0.4	[35]
Pioglitazone[b]	0.30	[61]
Pravastatin[d]	0.31	[61]
Quinidine[a]	0.76	[46]
Repaglinide[b]	0.49	[61]
Rifabutin	0.9	[36]
Sildenafil	0.9	unpublished data
Simvastatin	0.99	[46]
Tacrolimus	0.99	[36]
Terfenadine	0.74	[35]
Trazodone	0.35	unpublished data
Triazolam	0.92	[46]
Zolpidem	0.6	unpublished data
Zopiclone	0.95	[61]

[a]Significant renal clearance. [b]CYP2C8 substrates. [c]CYP2D6 substrate. [d]Transporter substrate. [e] N-glucuronidation

A number of approaches can be employed to estimate fm_{CYP} value. The most unequivocal method is based on the comparison of phenotyping data in extensive and poor metabolisers as shown for CYP2D6 [59]. A good alternative to this approach is 'phenocopying' based on the difference between the urinary recovery of metabolites in the presence and absence of a selective inhibitor (e.g., tolbutamide in the presence of sulphaphenazole as an inhibitor). CYP3A4 is the most problematic enzyme and the combined information on the urinary recovery of metabolites, biliary excretion and the recovery of unchanged drug has been used recently [36, 37, 47]. Table **2** shows the fm_{CYP3A4} estimates for 28 different drugs, ranging from 0.31 to 0.99 in case of pravastatin and buspirone, respectively. Recent study by Youdim *et al.* [42] has reported the use of *in vitro* fm_{CYP} estimates for the prediction of a range of ketoconazole DDIs. However, cautious interpretation of this *in vitro* approach is required due to the limited number of clearance processes (i.e., CYP mediated) measured and lack of information on the potential contribution of renal clearance to drug elimination.

Prediction of Time-Dependent Drug-Drug Interactions

In contrast to reversible inhibition, enzyme activity lost from time-dependent (mechanism-based) inhibition can only be restored by synthesis of a new enzyme. The rate of change of active enzyme concentration is determined by the equilibrium between the rates of de novo synthesis and degradation of the enzyme. Previous predictions of TDI [52, 61] used CYP3A4 degradation half-life estimates ($t_{1/2deg}$) obtained in either rat or Caco-2 cells [65], resulting in $t_{1/2deg}$ of 14-35 hrs. Recently, the estimates of human CYP3A4 k_{deg} from both induction studies or from *in vitro* investigations in liver slices have been collated [36, 54]. The estimated decay of CYP3A4 activity is 2-fold longer in comparison to most other cytochrome P450 enzymes [54] but comparable to CYP2D6 [66, 67]. The differential degradation half-lives reported for CYP3A4 and CYP3A5 *in vitro* (79 vs. 35 h, respectively) [66] and the lesser susceptibility to inhibition observed for CYP3A5 [68] may contribute to the extent of inter-individual variability observed in the magnitude of interactions.

Venkatakrishnan and Obach [67] have indicated the importance of accurate enzyme degradation estimates for the prediction of CYP2D6 inactivation by paroxetine; a recent comparable study was reported for CYP3A4 [36]. The impact of inter-individual variability of human CYP3A4 $t_{1/2deg}$ (20-146 hrs) on the assessment of TDI potential was assessed in conjunction with fm_{CYP3A4} considering their impact on the DDI prediction accuracy [47, 59, 64]. The sensitivity of the predicted DDI to a differential CYP3A4 degradation

rate was dependent on fm_{CYP3A4}. The prediction accuracy was very sensitive to CYP3A4 degradation rate for substrates mainly eliminated by this enzyme ($fm_{CYP3A4} \geq 0.9$), minimal effects are observed when CYP3A4 contributes less than 50% to the overall elimination in cases when the parallel elimination pathway is not subject to inhibition. The study also indicated the suitability of the mean CYP3A4 $t_{1/2deg}$ of 3 days in the assessment of time-dependent interaction potential.

The recovery half-life and degradation rate constant of CYP3A4 in the intestine can be estimated from grapefruit juice interaction data. The intestinal CYP3A4 k_{deg} of 0.00048 min^{-1} is reported resulting in the estimated CYP3A4 recovery half-life of approximately 24 h ([39], Gertz *et al.* submitted for publication], shorter than hepatic estimates.

Impact of the Intestinal Metabolic Interactions

Another confounding factor in the prediction of DDI is the possibility of inhibition at the level of the gut wall for some CYP3A drugs [69-72]. The contribution of an intestinal interaction is incorporated into the prediction equation based on the hepatic enzyme interaction as the F_G ratio; this approach is applicable for both reversible and irreversible inhibition interactions, as shown in the equations 6 and 7, respectively [40, 41, 61, 73]. So far, the incorporation of the intestinal inhibition into the DDI prediction strategy has shown variable success [35, 36, 40, 45, 61]. Initial investigations into incorporating maximal intestinal inhibition ($1/F_G$) did not improve prediction precision and accuracy of a range of reversible CYP3A DDIs [45]. In the case of time-dependent inhibition, Galetin *et al.* [36] have investigated the impact of the intestinal inhibition on 9 victim drugs (28 DDIs), with F_G ranging from 0.21 to 0.98 in case of buspirone and alprazolam, respectively. Although the use of the $1/F_G$ approach minimized the number of false negative predictions in this dataset, the number of predictions within 2-fold of *in vivo* value was reduced by 20%. Prediction of TDIs with midazolam, triazolam and nifedipine as victim drugs was generally improved by the incorporation of an intestinal interaction, whereas pronounced over-predictions were observed for the interactions with cyclosporine and buspirone (Fig. **6**). This is in agreement with a reversible DDIs database, where a similar trend was observed for atorvastatin and tacrolimus interactions [45]. Other studies have incorporated intestinal inhibition in the form of the predicted F_G ratio rather than assuming maximal inhibition [35, 40]. Obach *et al.* [40] have reported an improvement in the prediction precision and accuracy for a range of reversible CYP3A4 DDIs and also time-dependent DDIs [39] using unbound hepatic input concentration; however, when total hepatic input concentration was used as a surrogate for inhibitor concentration, the impact of intestinal inhibition was minor. Differences in the success of the prediction cannot be associated only with the incorporation of the intestinal contribution, as other parameters were inconsistent between the datasets (e.g., $fm_{CYP3A4,}$ use of average or hepatic input concentrations, enzyme degradation constants) for which the model is particularly sensitive [47, 64].

A potential for significant interaction in the intestine is associated with the relative ratio of intestinal concentration of the inhibitor to its estimated potency, i.e., I_G/K_i. Impact of physiological variability in enterocytic blood flow, inhibitor (I_G/K_i) and victim drug properties (control F_G) on the F_G ratio has been reviewed recently [60]. The F_G of a victim drug is an important determinant of the interaction magnitude. A minimal 50% intestinal extraction is indicated as an appropriate cut off value for a potential significant interaction, irrespective of the potency of the inhibitor and its inhibition mechanism [60]. CYP3A substrates with high extent of intestinal first-pass extraction (>75%) are particularly sensitive to any inaccuracy in the initial F_G estimates. Different methods to estimate the extent of intestinal extraction have been discussed recently [73]. For the drugs with intestinal extraction ≤ 50% (e.g., midazolam), the maximal value of the F_G ratio is approaching 2 even in the case of complete inhibition. This suggests that the contribution of the intestinal inhibition interaction is likely to be relatively minor; however, for some DDIs the 2-fold increase in the prediction may eliminate false-negatives.

Importance of Multiple Inhibitors and Multiple Inhibition Mechanisms

The contribution of multiple inhibitors (or metabolites) and/or the consequence of multiple inhibition mechanisms is rarely included in DDI assessment [36, 41, 74]. When two inhibitors act on the same enzyme and via the same mechanism, the effect of the least potent inhibitor in the prediction model is minor, in particular if the relative ratio of I/K_i of two inhibitors is > 100-fold [62]. Therefore, in such cases it would be sufficient to include only the

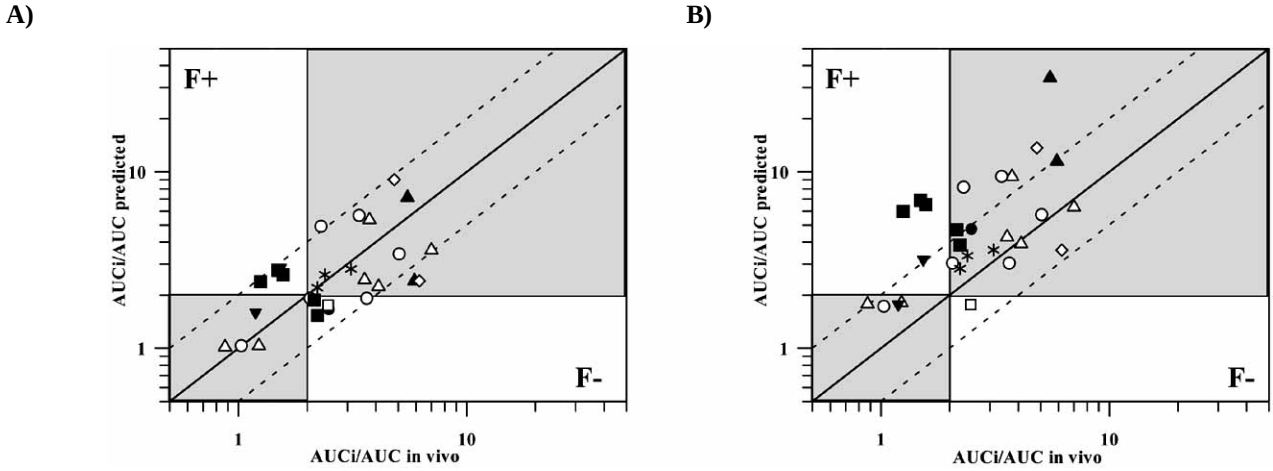


Fig. (6). Relationship between predicted and observed AUC ratios for time-dependent interactions.
A. Predictions of 28 TDI using Eq. 7 applying the average unbound plasma concentration of the inhibitor, corresponding fm_{CYP3A4} and CYP3A4 $t_{1/2deg}$ of 3 days and no intestinal interaction. Interactions identified according to the substrates: △ represents midazolam, ○ triazolam, □ alprazolam, ▲ buspirone, ▼ quinidine, ◇ simvastatin, ■ cyclosporine, ● felodipine and ∗ nifedipine. B. Effect of incorporating the maximal intestinal inhibition (F_G'=1) in the predictions; all other conditions and symbols are as in panel A. The solid line represents line of unity and dashed lines represent the 2-fold limit in prediction accuracy. The shaded areas correspond to the true negative and positive time-dependent interactions defined by the 2-fold increase in the AUC; F+ and F- represent false positive and false negative predictions, respectively [36].

more potent inhibitor in the prediction model. There is a perception that a greater combined effect is expected for inhibitors that act via different pathways (P450s) or independent mechanisms (e.g., reversible and irreversible as in case of gemfibrozil and its acyl-glucuronide). However, Hinton *et al.* [62] has shown that in cases when the contribution of the inhibited second enzyme is less than 50% (e.g., CYP3A in case of repaglinide), its contribution to the overall magnitude of the DDI observed will be relatively minor. However, inhibition of one P450 enzyme by a potent inhibitor (e.g., CYP2C8 by gemfibrozil glucuronide) may alter the fm_{CYP} balance towards a different P450 (e.g., CYP3A), which may explain the substantial inhibition effect observed in the itraconazole-gemfibrozil interaction with repaglinide [75] and loperamide [76], in contrast to minor interaction when only itraconazole is administered with repaglinide. In addition, disease- and age-specific differences in demography and differential metabolic activity of polymorphic enzymes will contribute to the inter-individual variability in the magnitude of DDIs with multiple inhibitors/inhibition mechanisms [41].

A number of recent studies [77-80] have indicated the importance of the hepatic uptake transporter OATP1B1 on the disposition and efficacy of a wide range of therapeutically used drugs, including statins, fexofenadine and repaglinide [63, 81-84]. Therefore, inhibition of the hepatic uptake of victim drugs may contribute towards the magnitude of observed DDI. Prediction of DDIs occurring via hepatic transporter proteins is currently based on an approach analogous to the basic metabolic model using the $[I]/K_i$ ratio [63]. This approach assumes that transport occurs exclusively via the particular transport protein subject to inhibition and that no passive uptake occurs and this approach is currently recommended by FDA in the ranking of potential transporter-mediated DDIs [46]. A recent study by Hinton *et al.* [62] proposed a refinement of this basic transporter model with the incorporation of a substrate-specific property. This additional term, the fraction of drug transported by a particular transporter protein (ft), is analogous to fm_{CYP} in the metabolic prediction model and allows differential contribution of the transporter of interest to the overall uptake. The impact of this victim drug-specific parameter on the prediction of transporter-related DDIs illustrates that even minor changes in ft (i.e., changes in transporter contribution from 100 to 80%) have a significant effect on the predicted AUC ratio in a similar manner to the effect of fm_{CYP} on the metabolic models. However, estimation of ft value is not straightforward. One approach is to estimate the contribution of each transporter to the total hepatic uptake *in vitro* using a relative activity approach [63, 85]. Alternatively, the OATP1B1 pharmacogenetic studies allowing comparison of AUC in transporter variants may represent a potentially useful approach to estimate ft, analogous to estimation of fm_{CYP2D6} from poor and extensive metabolisers [59].

Mechanisms for False Negatives and False Positives

As a result of the analysis described above, together with other publications in this area, the potential mechanisms for falsely categorising a potential DDI based on *in vitro* data can be summarised as follows:

False negatives. This may result from unrecognised TDI inhibition mechanism as well as an unrecognised contribution from inhibitory metabolites. A third potential source of false negatives would be the existence of transporter involvement which may lead to either incorrect values for K_i or the inhibitor concentration available to the enzyme.

False positives. The most common mechanism for this is likely to be an incorrect assignment of the fm_{CYP} value. As this is a very difficult parameter to estimate this is undoubtedly a very common shortcoming. This explanation would also explain some of the inconsistencies in different analyses in the literature of the same *in vivo* data.

False negatives and false positives. In addition there are some mechanisms, which may result in either a false positive or negative conclusion. This would include inhibition of CYP3A4 when the K_i values are substantially influenced by multisite binding, therefore the possibility of too high or too low K_i value can arise. Similarly the involvement of transporters may create an inhibitor concentration within the cell that differs from that in the medium (plasma) and the resulting effects may not be intuitive.

PERSPECTIVES FOR FUTURE APPLICATION AND IMPROVEMENT OF PREDICTION STRATEGIES

There are now several independent demonstrations of good correlations in the literature for both, prediction of clearance and DDI potential from both microsomes and hepatocytes. In the case of clearance prediction the value obtained is an absolute value; therefore there is a clear concern over the availability of good donor tissue and whether this is representative of the *in vivo* case being predicted. In contrast to clearance, DDI prediction involves relative values, which in theory is less demanding; however, the algorithms involved are multi-factorial and dealing with various parameters is often of uncertain precision. In the literature it is clear that there is inconsistency over the relative importance and the value of certain key parameters. Unfortunately there is a large subjective element in the use of these algorithms hence the author's opinion is paramount and is not always supported by sufficient documentation. Hence the uncertainty over which [I] is appropriate (both theoretically and pragmatically) for successful DDI predictions. While substantial advances have been made in the ease of generation of *in vitro* parameters, fuelled by the need for high throughput procedures and the general acceptance of generic protocols, these modelling aspects have received much less attention. However, the realisation that *in vitro* data *per se* is of limited predictive value is becoming more widely appreciated. The availability of simulation programmes (e.g. Simcyp®) is assisting the better use of *in vitro* data for prediction purposes.

Current trends in the type of new chemical entities under development as new drugs have necessitated a broadening of the scope of *in vitro* systems, and hence prediction strategies. In terms of metabolic stability it is now necessary to expand beyond cytochrome P450 and incorporate Phase II reactions as well as possibility of transporter uptake. The latter can be the rate limiting step in the hepatic clearance process [86]. Also the proteins responsible for these clearance processes are expressed extrahepatically and hence prediction strategies need to be expanded, e.g. to include extrahepatic metabolism, particularly in the gut and in the kidney, incorporating not only P450, but also UGT and SULT enzymes. In the commonly used liver models the term intrinsic clearance relies on the fact that clearance occurs via a number of parallel (usually metabolic) processes hence individual clearance terms are additive. With the involvement of transporters we have a hepatic clearance involving sequential processes rather than parallel ones and this may present some challenges for the future. It seems likely that the use of hepatocytes will increase to allow at least simultaneous measurement of these various processes and account for their spatial arrangement. However, the incorporation of the role of transporters is severely limited by the lack of information on the expression levels in the various tissues of the body and appropriate scaling factors. A combined physiologically-based model incorporating both the hepatic uptake transporter and metabolic pathways in a sequential manner would represent the most comprehensive prediction tool and the need for such refinement is becoming increasingly evident.

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